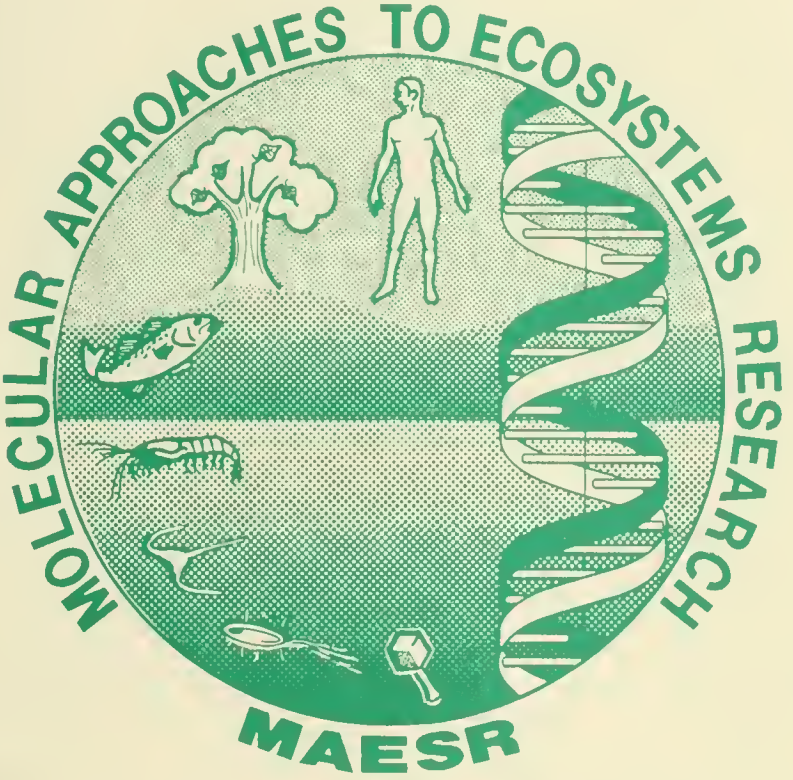
DOE/ER-0566T

MOLECULAR APPROACHES TO ECOSYSTEMS RESEARCH (MAESR)

An New Initiative for the U.S. Department of Energy



Based on a Workshop held at Asilomar, California
January 6-10, 1991

U.S. DEPARTMENT OF ENERGY

ENVIRONMENTAL SCIENCES DIVISION
OFFICE OF HEALTH AND ENVIRONMENTAL RESEARCH
and
ENERGY BIOSCIENCES DIVISION
OFFICE OF BASIC ENERGY SCIENCES

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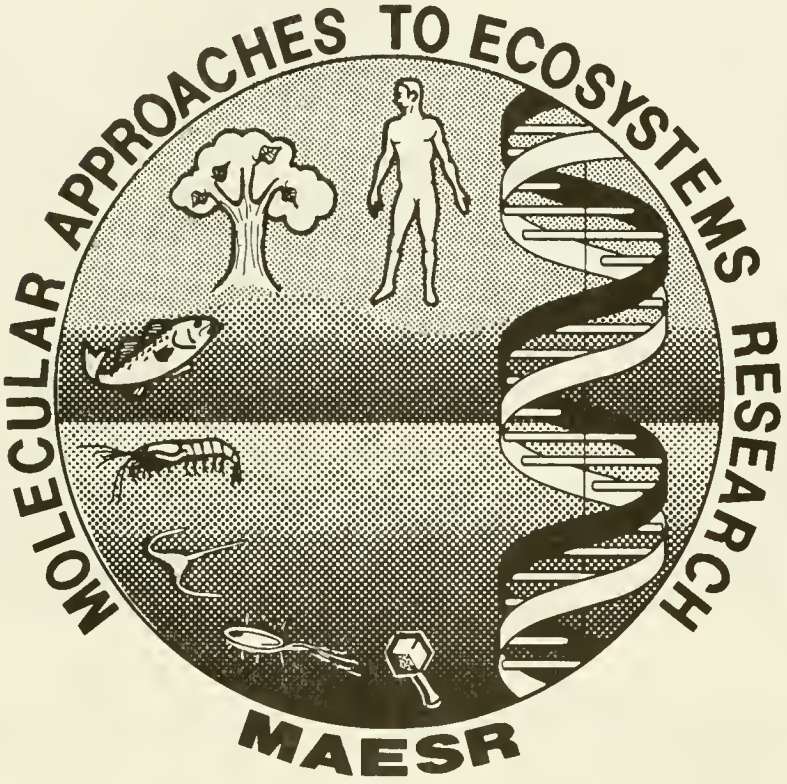

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WASHINGTON, D.C. 20585

Preface

The Department of Energy has accelerated integration of molecular biology into its current programs in ecosystems research, in order to better detect, understand, and predict the responses of ecosystems to environmental changes. This effort is not predicated on simply finding uses for the remarkable new tools of molecular biology. Rather, the effort will focus on the stated goals and objectives of existing programs, using molecular biology to facilitate and enhance the research.

In FY 1993, the Environmental Sciences Division of the Office of Health and Environmental Research (OHER) will enhance molecular biology research in three of its fundamental, interdisciplinary programs, Ecosystems Research, Ocean Margins, and Subsurface Science. Emphasis will be given to molecular biological methods as one of a suite of approaches that are appropriate for multidisciplinary research; techniques development will receive less attention than new applications of promising techniques. Specifically, the Ocean Margins Program will focus on the carbon cycle, the Subsurface Science Program will investigate the origins of microorganisms in the deep subsurface, and the Program for Ecosystems Research will focus on organismic and ecosystem adjustments of plants and soil microbiota during environmental changes. These three areas of research focus will add exciting new dimensions to our understanding of microbial origins and of microbial and plant function in natural terrestrial and aquatic habitats. They will also contribute to our understanding of how habitats are affected by energy exploration and use and help provide predictive and ameliorative capabilities in ecosystem management.

The following report, Molecular Approaches to EcoSystems Research (MAESR), represents a first step in the development of an ecological research program that fully integrates molecular biological methods and approaches. In the next few years, molecular biology will assume an even more prominent role in ecosystems research.

I wish to take this opportunity to thank Dr. Paul Falkowski for organizing and chairing the workshop that lead to the MAESR report. I am also grateful to Dr. Falkowski and his colleagues at Brookhaven National Laboratory for creatively weaving the ideas and working group reports that came out of the workshop into this excellent report on the future use of molecular biology in ecosystems research.

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Environmental Research, Office of
Energy Research

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Executive Summary

Energy extraction and use potentially have major effects on the global and local environment. As part of its mission, the Department of Energy's Office of Energy Research must understand the long-term effects of these activities. To accomplish this mission, a scientific research program of the highest quality is required which addresses the question of how energy activities affect both global climate and major ecological processes. It also is increasingly critical that the scientific program lead to an unprecedented predictive and anticipatory capability. While the assessment of damage and change is useful and important, understanding key processes which provide a predictive capacity will be much more useful in developing a responsible policy for energy extraction and use in the 21st century.

In concert with other Federal agencies, and through the Committee on Earth and Environmental Sciences (CEES), DOE has developed and fostered long-term research to understand how specific ecosystems respond to and affect the global physical and chemical environment. Recently, DOE participation in the Biotechnology Research Subcommittee (BRS) of the Federal Coordinating Council for Science, Engineering, and Technology (FCCSET) has initiated a similar commitment to long-term research in molecular biology. An understanding of many critical processes requires knowledge of key biological regulatory mechanisms and responses at a molecular level. The large-scale processes of ecosystems basically depend on the small-scale responses of their constituents. Recent advances in molecular biology have provided fundamental understanding of living organisms, as well as new experimental tools which have not been fully exploited to advance our understanding of ecosystems. Therefore, **DOE proposes to integrate the techniques of modern molecular biology into its ecosystems research programs. The specific focus will be on detecting and understanding the responses of ecosystems to environmental changes. Such information is crucial to interpreting and predicting the effects of energy-related activities on the biosphere, and their feedback on future climate.**

The Molecular Approaches to EcoSystems Research (MAESR) report was developed after a workshop held on 6-10 January 1991 at Asilomar, California. The report identifies three major areas of opportunity in which the application of molecular biological

techniques could substantially advance our understanding of ecosystems and their responses to environmental changes.

1. Identifying the factors limiting and regulating the biologically driven fluxes of geochemical elements.

2. Understanding the important processes that determine the ability of organisms to adapt physiologically to environmental changes.

3. Assessing the impact of chronic, long-term environmental changes on the stability, diversity, and function of biological communities.

The report provides a strategy to foster the application of molecular techniques in ongoing multidisciplinary research in terrestrial, aquatic, and marine ecosystems. The initiative will cut across DOE's programmatic elements, and will be implemented through competitive research grants, post-graduate fellowships, and training grants; the initiative will promote interdisciplinary research between ecological and molecular biological sciences. Additionally, implementation of the MAESR report will provide basic scientific information which will improve efforts in bioremediation, site clean-up, renewable-resource production, and waste management.

Introduction to Ecosystems

All organisms depend directly and indirectly on the activities of other organisms for their survival and growth. The relationships between diverse, and often seemingly unrelated, organisms have evolved over millions of years to higher-order complexes, called ecosystems. Ecology is the study of such ecosystems. The goal of ecology is to understand and quantitatively describe the fluxes of materials and energy between functional groups of organisms, how the functions of these groups affect the chemical and physical environment, and how the environment, in turn, affects the function and structure of the ecosystem.

The Role of Molecular Biology in Ecology

Because biological processes modify the chemical and physical environment, understanding how ecosystems function is crucial in predicting the effects of potential change on the future environment. Fundamental insights into how organisms function, respond to short-term change, and ultimately evolve, lie in their genetic material. A thorough understanding of ecosystems requires a knowledge of molecular biology, the study of the function and regulation of genes and their products. When integrated into higher levels of ecological research, molecular biology can provide basic information on the regulation and mechanisms of key ecosystem processes. Such information is crucial in developing predictive models of how ecosystems will respond to energy-related activities on global and local scales.

The Scaling Problem

Darwin's theory of evolution by natural selection, developed in the mid 19th century, provided the conceptual basis for ecosystem research. The elucidation of the structure and function of DNA by Watson and Crick in the late 1950s was a watershed for modern molecular biology. While biologists basically understand that gene function and regulation are ultimately related to how ecosystems evolve and function, integration of that information into ecological understanding has been slow, primarily due to compartmentalization and specialization within biological subdisciplines.

Molecular biological and ecosystems research spans up to 15 orders of magnitude, temporally and spatially (Fig. 1). This biological space-time scale has an analogue in that expanse between particle physics and astrophysics. Here, a fundamental understanding of physical processes on subatomic scales is required to explain the behavior of immense celestial

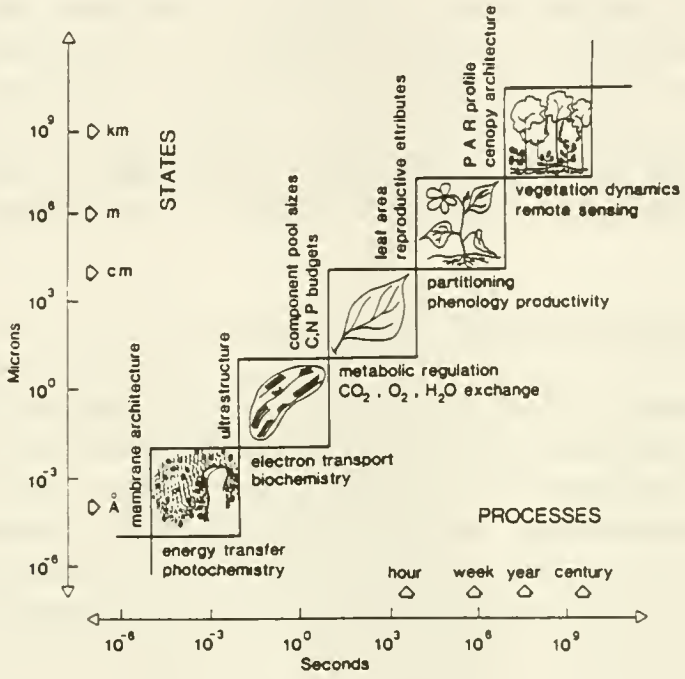

Fig. 1. An example of the scales of magnitude differences between systems ecological processes and molecular biological processes. In photosynthesis, light harvesting and primary charge separation, which comprise the actual process, occur on spatial scales of ca. 10^{-5} to 10^{-4} microns and time scales of 10^{-5} to 10 seconds. These processes are strongly tied to molecular events through the synthesis and assembly of the photosynthetic apparatus. The integration of the photosynthetic apparatus into the overall metabolic processes of higher plant occurs on spatial scales of ca. 10^3 microns and time scales of 10^3 seconds, and on plant growth, on scales of ca. 10^6 microns and time scales of 10^6 seconds. The ecosystem scale, of 10^9 microns with a time scale of 10^9 seconds, is, in turn, an integral of the molecular, metabolic, and reproductive processes on a community level. (From Osmond et al., 1991). Associated with each space-time scale are specialized disciplines of biological research; at present, disciplines at the small scales are poorly integrated into ecosystems research.

objects. Physicists also recognize that natural astrophysical phenomena, such as black holes, provide a potential basis for developing and testing new theories of the fundamental behavior of matter. The temporal and spatial scale differences between ecology and molecular biology are huge, but integration of molecular biological processes into ecosystem models eventually will comparably enrich these two most important biological fields.

The Key Research Areas in Molecular Ecology

The DOE sponsored an interdisciplinary workshop in Asilomar, California, from 6-10 January 1991, to identify key areas where molecular biology could quickly provide the most useful information for understanding ecosystems. The workshop, entitled "The Molecular Bases of Ecology," brought together about 60 scientists, including terrestrial and marine ecologists, microbiologists, molecular biologists, biophysicists, and physiologists. Participants concluded that the following three areas of ecosystem research would benefit from molecular research:

1. **Identifying and quantifying factors limiting and regulating the biologically driven fluxes of geochemically important elements, especially those related to climate.**

2. **Understanding the processes that determine the ability of organisms to physiologically acclimate and genetically adapt to environmental changes.**

3. **Assessing the impact of long-term environmental changes on the stability, diversity, and function of biological communities.**

Unlike traditional, controlled laboratory experiments, where the influence of one variable on a process can be described independently of the others, ecosystems research is largely multivariate (and often, like astrophysics, uncontrolled). Inferences must be drawn, either statistically, by modeling, or by manipulating the environment, often without completely understanding the regulatory processes. **Molecular techniques offer the possibility of interrogating the status and trends of individual organisms, populations, or communities in the natural ecosystem without manipulating the environment.** Using the genetic information of an organism enables that organism, in effect, to become its own reporter. Interpretation of that genetic

information is a challenge for molecular biologists, but the realization of that goal will provide systems ecologists with a more complete understanding of biological processes and how they will be affected by environmental changes. Ultimately, to effectively integrate molecular biology into ecological research, molecular biologists will need to develop an understanding of crucial ecological processes and transfer their laboratory based skills and techniques to the field. **A major goal of this initiative is to foster interdisciplinary collaboration, thereby promoting the transfer of those skills and providing a means to integrate scientific disciplines.**

Current Themes and Issues in Systems Ecology

A goal of ecosystems research is to quantify the fluxes of materials and energy between trophic levels. Many key elements in biogeochemical cycles undergo reduction and oxidation as a result of biological processes. Oxidation-reduction reactions fuel all biological processes and are the major classes of biochemical reactions affecting or affected by the composition of the atmosphere. A major biological reduction process is photosynthesis, in which carbon dioxide is biochemically reduced to organic carbon. The major reductant is water. A major oxidation reaction is aerobic respiration, in which organic carbon is chemically oxidized, providing energy and liberating inorganic carbon. The oxidant is molecular oxygen. Other key elements, such as nitrogen, sulfur, iron, and manganese, also undergo biologically mediated oxidation-reduction. The cycles of these elements, and others, such as phosphorus (which is not oxidized or reduced by biological activity), ultimately regulate the carbon cycle.

The workshop participants identified three basic areas of ecological uncertainty in understanding the regulation and function of biogeochemical cycles:

(1) **The characterization of external, rate-limiting processes** in natural ecosystems.

(2) **The characterization of inherently imposed energetic constraints on the ability of organisms to grow and function.**

(3) **The characterization of the relationships between community structure and environmental perturbation.**

The workshop examined the potential application of molecular biological approaches to help systems ecologists quantify and identify limiting factors, energetic constraints, and community structure and stability. Here, we briefly discuss some of the issues faced in understanding how these themes affect the dynamics of global biogeochemical cycles, especially those affected by energy-related activities.

Limiting Factors

In nature, population density and the growth rate of organisms may be significantly less than the maximum rate under optimal conditions. Environmental constraints, or limitations, directly affect biogeochemical cycles. For example, photosynthetic organisms, which reduce inorganic carbon to organic materials, may be limited by the availability of nitrogen, phosphorous, carbon, or iron, and by the presence of natural or anthropogenic toxins, or by physical factors, such as light, temperature, and salinity. Heterotrophic organisms, which oxidize organic carbon compounds and contribute to the production of CO_2, may be limited by the availability of organic carbon, nitrogen, or temperature, or by physical factors. Fundamental understanding of ecosystems requires the identification of the factors limiting key biogeochemical processes. Ecologists have traditionally identified limiting factors by experimentally manipulating the organisms or small portions of ecosystems, or by correlative inference. Often these approaches are extremely tedious, expensive, ambiguous, and contentious, and provide little predictive capability; at present, there are few alternative <u>in situ</u> techniques. Thus, there would be a major breakthrough in understanding ecosystem dynamics and biogeochemical cycles if we could identify and understand the rate-limiting factors for key biogeochemical processes in specific ecosystems.

The research would entail the following two elements:

(a) identification of specific diagnostic genes or gene products indicative of a limiting factor; and

(b) identification of the mechanisms or regulatory motifs by which organisms perceive the environment and transduce that signal to modify the expression of specific genes or gene products.

Two specific areas where an understanding of rate-limiting processes is crucial to the global carbon cycle are terrestrial and oceanic photosynthesis. In terrestrial ecosystems, the effects of both changes in temperature and of the availability of water and CO_2 on higher-plant production are largely unknown. On a global scale, water is the major determinant of the distribution and production of terrestrial plants. Climate models suggest that a major effect of long-term alteration of the radiation balance in the atmosphere will be on the hydrological cycle. The ability to withstand drought is highly variable among higher plants. Drought stress induces changes in growth rate, morphology (root/shoot ratios), physiology (leaf conductance), and gene expression. The types of changes depend on the stage of the life cycle of the plant, and the severity and duration of water deficit. Genes encoding channel-forming proteins, a thiol protease, and enzymes involved in osmotic adjustment are induced by a mild water deficit. Protection from more severe dehydration is correlated with the accumulation of a specific set of hydrophilic proteins, the dehydrins, whose exact function is unknown. The genes which encode these proteins are induced by the hormone abissic acid (ABA), which increases in tissues that are partially dehydrated. The DNA elements which mediate responsiveness to ABA and the proteins which bind to the ABA, cis-elements, have been identified. In situ measurements of proteins, and their mRNAs, will be diagnostic markers of water stress and will provide an understanding of how these genes are regulated in nature.

The effects of increased CO_2 levels on plant growth may also be beneficial; CO_2 itself may limit photosynthesis in terrestrial plants. In natural ecosystems, secondary limiting factors, such as potassium, sulfur, or nitrogen, may retard or eliminate any increase in production resulting from CO_2 fertilization. These factors have specific molecular diagnostic markers, which may reveal the limitation of carbon fixation by plants in nature. Little is understood about the overall short- and long-term coordinate adaption of plants to various changes in nutrients and other environmental conditions.

About 50% of global photosynthesis occurs in the oceans. Identifying the factors which limit oceanic photosynthesis has occupied the attention of biological oceanographers for three decades. Traditional methods for determining the limiting factor

are either long-term incubation of water samples in bottles containing the suspected limiting elements, correlative inference from proximate chemical analyses, or mathematical modeling. The findings from such studies are often contentious. Most ocean ecosystem models relate carbon fixation to the availability of inorganic nitrogen; this seems to be applicable in most of the oligotrophic open ocean, where the concentration of inorganic nitrogen is vanishingly low. However, in the Subarctic Pacific and the Southern Ocean, there is excess inorganic nitrogen and phosphate; temperature, light and, more recently, iron have been suggested to be limiting factors. Despite extensive observations, ecologists are often still unable to unequivocally identify which of these potential factors is the rate-limiting one.

Several laboratory studies suggest that unique molecular markers, diagnostic of specific limiting factors, may be used to indicate the factor limiting growth in situ, and perhaps the degree of limitation. For example, in cyanobacteria, a set of membrane-bound proteins is synthesized in iron-deficient cells. In diatoms, iron deficiency appears to alter the migration of the small subunit of the carboxylation enzyme ribulose 1,5-bisphosphate carboxylase. Some phytoplankton species synthesize a chlorophyll protein (CP) complex similar to CP43, but lacking a 100 amino acid domain localized in the membrane lumen. The gene for the protein (which is highly conserved in all oxygenic photoautotrophs) is controlled by an iron-regulated promoter, which appears to be recognized specifically under iron deficiency. In situ measurements of these types of responses will provide a basis for examining the degree to which phytoplankton photosynthesis may be limited by iron in the ocean. Similar markers are known from laboratory studies on nitrogen and phosphorus deficiency, so that a sense of in situ molecular marker measurements may resolve which factor is rate limiting.

The growth rates of microbes in nature appear to be much lower than their potential capabilities. For example, estimated growth rates in aquatic systems are at least one to two orders of magnitude lower than those for laboratory cultivated microbes. Microbial activity is important for the regeneration of many of the substrates for photosynthetic organisms. Determining which factors limit microbial growth rates is essential to understanding the fluxes of many biogeochemically important elements. In laboratory studies, specific proteins and membrane lipids are induced in gram-

negative aquatic bacteria by substrate limitation. Organic carbon limitation induces proteins in the periplasm of these bacteria. A unique marker protein, indicative of low endogenous substrate levels, is found in the outer membrane of starved cells. Additionally, fluorescent-labeled antibodies have identified specific carbohydrates synthesized during starvation. These approaches are amenable to understanding the availability of substrates for natural microbial communities in situ.

A variety of other molecular biological, diagnostic indicators of physiological rate-limiting factors is described in Appendix I. There are known molecular markers for nitrogen limitation, elevated and depressed temperature, metal tolerance and toxicity, and responses to suboptimal and supraoptimal irradiance effects. Their ubiquity is poorly understood. **A major challenge for molecular biological research is to develop an adequate base of knowledge to interpret molecular signals from organisms in the natural environment, and to infer from such signals how, and to what extent, the growth and function of the organisms is limited by external forcings.**

Below are some examples of questions where the techniques of molecular biology could be used to understand limiting factors:

- How can laboratory studies that identify specific proteins or other molecular markers symptomatic of limiting factors or stresses be used in natural ecosystems to specifically screen and diagnose limiting factors?

- How can the so-called stress proteins, such as ubiquitin, heat-shock proteins, or their analogues, be used to diagnose thermal, water, and light stress in the field?

- How can starvation-induced proteins, known from laboratory studies of starved cells, be used to identify substrate limitation in mixed, natural microbial communities?

- Are specific proteins or secondary gene products, such as lipids, synthesized in response to small, sublethal changes in temperatures?

Signal Transduction Mechanisms

A fundamental issue in interpreting molecular signals on short time scales is how environmental cues are sensed, what the mechanisms are for signal transduction, and how these signals are extended to elicit gene expression. The physiological literature is rife with descriptions of how a variety of cues, including light, temperature, pH, salt, pressure, nutrient availability, and dissolved organic compounds, affect the growth, behavior, and reproductive responses in myriad organisms. Signal transduction mechanisms are found in procaryotes, as well as in higher organisms. In the evolution of transduction mechanisms, common genetic switches and switching mechanisms are likely to have been conserved. Signal transduction mechanisms have been isolated and described in one or two model systems; however, it is unclear how far these models can be extrapolated. **Therefore, a second aspect of using a diagnostic approach to interpreting how organisms are affected by the environment, requires a comparison of common regulatory and signal transduction motifs at the molecular level.**

Some key questions on signal transduction mechanisms that were identified are the following:

- Are the sequences or motifs in flanking regions on genes that code for stress-induced proteins conserved in a wide variety of organisms?

- How are transcriptional elements, or factors, activated by external chemical or physical signals?

- How are the activation processes reversed; i.e., how does environmental regulation of genes fundamentally differ from cellular differentiation? How are these two processes similar?

- How varied are the environmental sensing systems in natural ecosystems?

Energetics

Although the external physical and chemical environment controls many ecosystem functions, as was described briefly in the section on limiting factors, inherent metabolic processes also play

a key role in biogeochemical cycles. For example, inherent biological processes limit the maximum rate of photosynthetic energy-conversion in plants. Often, however, neither the rate-limiting step, nor magnitude of the rate-limiting process, is experimentally defined by classical techniques. Additionally, many organisms channel materials and energy through different pathways or into wasteful (futile) cycles. Futile or inefficient cycles lead to production of soil humus, refractory dissolved organic compounds in the ocean, and, over geological time (in conjunction with non-biologically mediated, geochemical processes) fossil fuel repositories. An understanding of how organisms respond to environmental changes by altering biochemical fluxes may eventually allow reasonable and predictable models to be made involving material and energy flux within major groups of organisms, and ultimately, between them and their ecosystem.

Understanding the molecular basis of inherent biological limitations of energy and material fluxes has the following primary goals:

a) to improve our understanding of the mechanisms and measurement of energy and material fluxes through food webs and the measurement of the magnitude and proportions of materials that are not recycled; and

b) to use these mechanisms (and the ecosystem models derived from them) to obtain information to determine how environmental or global change would affect pathways and magnitudes of the fluxes to the atmosphere and stored in non-reactive pools.

An understanding of how these inherent limitations influence the fluxes of materials and energy in natural ecosystems will require extensive laboratory studies with individual organisms and microbial consortia, as well as studies of field populations. Several levels of study will be needed, many of which lend themselves well to the application of molecular genetics, geochemistry, biophysics, and biochemistry. It was recommended, in general, that high priority be given to the isolation and characterization of causative organisms and their study as individuals and as active consortia.

The ideal method for determining inherent metabolic activity would have several key features. First, organisms would be

collected quickly, without fractionation and disruption of consortia, and would be fixed or otherwise "frozen." Second, the samples could then be stored long enough for transport to a laboratory; that is, it would not be necessary to carry out complex analysis in an inconvenient location (e.g., aboard ship or in a forest). Third, the method would analyze growth rates in single cells in a species or in a group-specific manner so that different growth rates by different components of a consortium or community could be discerned. Fourth, the method should be broadly applicable to a variety of organisms.

Two possible molecular approaches are given here as illustrations. The first example is an estimation of growth rates from the ratio of ribosomal RNA (or some measure of ribosomes) to DNA of single cells. Consider two fluorescent probes (which can be differentiated by emission wavelength); one hybridizes to the rRNA of a particular species or genus, and the other hybridizes to some chromosomal DNA segment. The sample containing the consortium or population is fixed to a slide, permeabilized, and then probed with the rRNA and DNA probes. Then, the fluorescence of the two probes is measured separately by fluorescence microscopy (a microscope fitted with a detector to quantitate light emissions and supporting image-processing software) or flow cytometry. The ratios of more than one type of organism could be measured simultaneously if more fluorescent labels were available. This type of approach could simultaneously estimate the growth rates of different members of a consortium while preserving the geometry of the consortium. **Developing and using new methods to elucidate metabolic activities and growth rates in nature is a high priority.**

A second approach would be to use the polymerase chain reaction (PCR) to detect messenger RNAs in cells and find mRNAs or proteins that could be used as indicators of growth. Preliminary evidence suggests that some mRNAs and proteins are expressed preferentially as bacteria shift from rapid to slower rates of growth. In eucaryotes, cell cycle-specific proteins are well documented. The activity of cell processes could be associated with such mRNAs and proteins, and probes and antibodies could be used in cross-sections or smears from natural samples.

A special subset of the issue of biochemical flux is that of the maintenance energy of cells: namely, that portion of the

metabolic budget of the cell that is concerned with survival and maintenance rather than growth. Such maintenance functions are usually thought of as, for example, the maintenance of osmotic gradients, repair of cellular damage, and transport of nutrients. Under many conditions, especially those of nutrient limitation or slow growth, maintenance energy is thought to be a major portion of the metabolic budget of cells. Alternatively, during long-term starvation, all metabolism, including maintenance energy, may approach non-functional (or at least non-detectable) levels. **This issue may be of central importance to our understanding of the role of the biota in the carbon cycle of many different environments.** For example, the importance of the microbial loop in aquatic ecology is assessed directly from estimates of growth rates of bacterial populations.

One of the tenets of the carbon cycle is that burial has accounted for a major geological sink for carbon on Earth. Such processes occur when refractory carbon is buried before it can be oxidized to CO_2, and is, thus, fixed or sequestered as sedimentary carbon. **Thus, understanding the processes that lead to the oxidation and burial of carbon are of central importance in understanding the global fluxes of carbon.**

Proceeding vertically downward through virtually any stratified environment, including lakes, fjords, ocean basins, marshes, marine and freshwater sediments, and even many soils and other terrestrial sediments, oxygen usually disappears as a major element, because of aerobic respiration and the resulting consumption of oxygen. Subsequently, other available electron acceptors are sequentially used, usually in a manner consistent with the thermodynamic energy available (e.g., nitrate is used after oxygen and followed by manganese, iron, sulfite, sulfate, and CO_2). If the rates of consumption or production of metabolic products are greater than their rates of diffusion, then redox boundaries are established, leading to boundaries between oxygen and nitrate, nitrate and manganese, manganese and iron. Across these boundaries, it is common to find high accumulations of microorganisms capable of more specific redox reactions. For most carbon- oxidizing anaerobes, there are methods to estimate reaction rates in the laboratory; however, there is a critical need to develop techniques which also can be applied to rate measurements in the field.

Current ecosystem models suggest that an increase in the global mean temperature will lead to a larger increase in respiration than in photosynthesis, primarily because of microbial metabolism. If so, the predicted effect would be to further increase atmospheric CO_2 levels, potentially leading to even higher temperatures: a positive feedback. The effect would be most dramatic at high latitudes in the northern hemisphere, where climatic models predict the greatest temperature change, and where significant amounts of organic carbon are deposited. The ecosystem models are poorly parameterized to analyze this response.

Molecular techniques are potentially available to examine how temperature will differentially affect photosynthesis and respiration. For example, if photosynthesis is not temperature limited, but limited by CO_2 availability, then increased temperatures would potentially lead to increased transpiration but decreased efficiency in water- use; e.g., effectively, a drought-stress response. The maximum rate of photosynthesis is internally limited at light saturation by the ratio of dark-carboxylation capacity to electron-transport components. Using protein immunoblotting methods in conjunction with non-invasive biophysical probes of fluorescence, the inherent limitation on photosynthesis in both higher plants and microalgae in nature can be studied. Such studies would provide a basis for understanding how temperature affects carbon fixation, providing that an excess of other potential external limiting factors is present. An increase in temperature would be expected to shift microbial communities slowly from psycrophyllic (cold-loving) to mesophyllic (moderate temperatures). This community shift might be tracked with molecular taxonomic techniques (see next section on Community Structure).

The following are examples of key questions that were identified with respect to inherent biological limitations related to the fluxes of materials and energy:

- What inherent processes determine the efficiency with which organisms oxidize or reduce carbon?

- How do various metabolic groups fractionate carbon, nitrogen, sulfur, oxygen, hydrogen, and phosphorus?

- How is isotope fractionation related to the primary structure of the rate-determining enzyme?

- What determines the upper rate of growth and production of an organism?

- Can specific molecular indices of growth be developed for eucaryotic as well as procaryotic organisms?

- How do biogeochemical processes related to metabolism change with the growth of organisms?

- How can we improve our measurements of metabolic activity of anaerobic organisms in natural ecosystems?

- How can we differentiate between maintenance energy and energy used for net growth in organisms in nature?

Some specific suggestions are described in Appendix II.

Community Structure

Each organism, or more precisely, each strain or species, occupies a niche, which may be conceived as a multidimensional phase space in which the organism lives and reproduces. For most organisms their potential niche, the portion of the phase space in which they could live, is much larger than their actual niche. Existence and reproduction outside the niche are impossible without genetic alteration.

Although the earth underwent two complete ice ages over the last 160,000 years, the composition of the atmosphere remained relatively constant. Because of this relative stasis, it was assumed that the relative rates of critical biogeochemical cycles also remained relatively constant. However, this does not mean that ecosystem structure has remained constant, because the organisms which were negatively affected by the climatic changes were selected against and eventually replaced by more functionally redundant organisms. However, the environmental changes forecast by the present global climate models are so fast, compared to the rate of previous climate changes, that it is unclear whether biotic components can adapt rapidly enough to maintain a relatively steady state in the ecosystem's functioning.

On long time-scales, of centuries to millennia, environmental changes affect the structure of biological communities within ecosystems by altering the species composition through natural selection. The functional stability of ecosystems includes species composition, functional redundancy among species, genetic diversity within species, and numerical abundance among the species. Changes in community structure and diversity due to natural and human perturbations may or may not have a measurable effect on ecosystem function. To understand the ecosystem changes, ecologists must be able to measure changes in the diversity, abundance, and degree of functional redundancy of organisms.

The classical model of ecosystem change following a disturbance is portrayed by successional changes among organisms that are driven by patterns of migration, competition, and environmental modification by alternative organisms. This process results in a dynamic community, usually assumed to be best adapted temporally and spatially. Over the past two decades, virtually every aspect of this model has been seriously questioned. Notions of long-term environmental stability have been shattered by paleoecological studies that reveal constant climatic change at temporal scales ranging from decadal oscillations to 100-millennia ice ages. In many communities, changes do not lead to biotic stability. Indeed, changes in ecosystems may increase the likelihood of disturbance, as occurs, for example, in forests which accumulate flammable fuels. The resulting cycles of disturbance may generate complex arrays of "metastable" community configurations.

For microbial communities within ecosystems, conceptual models of successional change are based primarily on competition for limited multiple resources. However, documenting succession, which is only the first step in understanding the ecosystem's dynamics and self-adaptation, has been severely hampered by the inability of ecologists to identify, enumerate, and characterize the myriad of microbes present. Only a small fraction (< 1%) of microbes that live in nature can be readily cultivated in the laboratory. Moreover, it has become increasingly clear that many microbial communities evolved with complex interactions, forming so-called consortia. Techniques of molecular biology have been especially promising in enabling microbial ecologists to identify and enumerate microbes, based on the characterization of nucleic acids,

without having to isolate and cultivate them individually. For example, using nucleic-acid sequencing, microbes can be identified from ribosomal RNA, as well as from genomic DNA. These techniques, described in Appendix III, are applicable to natural ecosystems with highly diverse microbial communities and consortia.

Several questions, set out below, are critical to determining the trajectory of ecosystem structure and function under environmental alterations. The answers provide critical information about how the biological systems will be affected by environmental perturbations, and will themselves affect the environment.

1. How does chronic stress alter species diversity?
   a) What rate of environmental change causes species diversity to change?
   b) What is the trajectory of change in species diversity as a function of increasing perturbance?

2. What is the relationship between functional redundancy and species diversity? What is the effect of stress on functional redundancy?

3. How will changes in environmental variance affect genetic diversity within a population?
   a) Is genetic diversity independent of environmental change or related to it?
   b) How is intraspecific genetic diversity affected by competition?
   c) How does intraspecific genetic diversity affect the rate of change in functional redundancy during stress?

Many of these questions can be addressed by molecular biological techniques; indeed, in many cases, molecular biology has had its greatest impact in ecology in elucidating community structure and diversity. Techniques which exploit pattern differences (polymorphisms) in nucleic acid restriction fragments (RFLPs), and the rapidity and ease of use of the PCR can be used to assay intraspecific genetic diversity. Comparative analyses of nucleic acid sequences, particularly of rRNA sequences, are revolutionizing our understanding of microbial diversity and phylogenetics. In addition, sequence analyses provide information for designing species or group-specific hybridization probes that

are especially useful for dissecting community structure, because they can identify species in mixed, natural communities without the need to subculture or grow the organisms in the laboratory. Nucleic acid hybridization techniques also can be used to identify and localize individual cells. Binding of probes in cells can be detected by epifluorescence microscopy or flow cytometry. The use of in situ probes has a great potential for elucidating the spatial relationships of microbial organisms which exist in complex, interdependent consortia. Immunological techniques also can identify organisms, either to species level or to a functional group level.

While the aforementioned techniques can be, and to some extent are being, used to objectively and rapidly document genetic diversity within natural ecosystems, the underlying causes and mechanisms of genetic change remain obscure. Resolving this issue is central to understanding how genetic change is related to environmental perturbation. Experimentally, the causality of genetic change is presently best addressed in a laboratory, where organisms can be manipulated to study factors which influence the rates of genetic mutation and how these rates affect community stability. As environments change, there is a need to know how populations, both starved (dormant) and actively growing populations, adapt genetically and how this adaptability is related to the functional resistance or resilience of the populations.

Thus, the molecular focus of questions related to community structure and stability is primarily on the causes and effects of external environmental factors, and how they affect genetic change. Molecular biological techniques can rapidly and precisely detect mutations; however, it is unclear how the natural rate of mutation and selection can be differentiated from an environmentally accelerated rate.

There is some indication that chronic environmental stress can increase the mutation rate in some microbes. This effect could be important in increasing the adaptability of populations by increasing genetic variability. The mechanisms for this phenomenon are obscure; although it has been well documented in model organisms such as Escherichia coli, it is unclear how far the findings can be generalized. Is there historical evidence for increased genetic variability in response to environmental change? For example, pollen DNA from extant species can be assayed for

genetic variability over the last 10,000 years, and compared with the records of changes in CO_2 and temperature. The genetic variability could be determined by the RFLP technique or by more conventional PCR with restriction site mapping, focusing on the relationship between the rate of environmental change and mutation rate.

Molecular biological methods for describing community structure are one of the best approaches for elucidating community diversity, and it is essential that they be incorporated into ecosystem programs.

Integration of Molecular Biology with Other Disciplines

The integration of molecular biology into ecology can be brought about only by disciplines which bridge or contribute to these two areas of biology; these include, for example, biochemistry, physiology, microbiology, genetics, population biology, and geochemistry. The MAESR initiative is designed to supplement and develop, not replace, these bridging disciplines. For example, stable isotope fractionation studies are invaluable for integrating biological and geochemical processes; molecular biology in conjunction with biochemistry can clarify the mechanisms and variability of the fractionation process. Variations in fluorescence yields of plants and phytoplankton can be used to infer stress or productivity in the field. Molecular biology and physiology provide an understanding of the molecular bases of the variations.

Relationship of MAESR to DOE Goals

MAESR was conceived as a means of improving the reliability and predictive capability of ecosystem models by including a mechanistic understanding of how biological processes operate. It was recognized at the workshop that DOE's interests in bioremediation and biotechnology would also benefit from MAESR. The molecular biological approaches described for understanding biogeochemical cycles can easily be applied to understanding the function of deep subsurface microbial communities, potentially altering natural microbial communities to selectively absorb radionuclides or other substances from complex mixtures, and developing better biological processes for degrading noxious

compounds. Thus, DOE programs related to site clean-up, production of renewable resources, and waste management also will benefit from the MAESR report.

Significance of MAESR

Basically, the key themes in ecosystem research were developed early in the 20th century, yet progress in understanding how ecosystems function has been slow. For the most part, ecologists have applied techniques developed in other areas of science. Large leaps in understanding have come from the application of new techniques. Over the past 20 years, for example, satellites have provided a capability to document and quantify many ecologically relevant processes on a global scale. Improved technology and long time-series analysis have facilitated the accurate measurement of extremely small changes in atmospheric gas composition, and the inference of globally integrated biological processes such as respiration and photosynthesis. Improved computational algorithms and hardware have led to the development of relatively sophisticated numerical simulation models, which can be integrated into general atmospheric circulation models (GCMs) to examine the feedbacks between biological processes and climate. At present, however, a mechanistic understanding of how ecosystems function and respond to change is lacking.

MAESR will provide, for the first time, a comprehensive molecular biological approach to understanding systems ecology. The questions posed in the report were developed primarily by ecologists. The alteration of the chemical composition of the biosphere represents anthropogenic, global selection pressure, whereby organisms may be forced to extinction, without our clearly understanding the consequences. Ecologists need to quantitatively predict how that selection pressure will affect the living resources of the earth on short and long time scales, and how those effects will modify the environment. MAESR, described here, provides a framework for that understanding. Implementation of this initiative will provide new scientific challenges for molecular biologists, leading to stronger interdisciplinary research efforts in understanding how energy extraction and use will affect the major ecosystems of the world.

Appendix I
Molecular Approaches to Understanding Limiting Factors

Understanding the factors limiting biogeochemical cycles is crucial to predicting how changes in the physical and chemical environment will affect ecosystem responses. Although many regulatory processes have been well characterized at the organismal and biochemical levels, we know much less about molecular mechanisms in nature and how they are regulated by the present global environment. The carbon and nitrogen cycles have been singled out as examples, although cycling of other elements and their interactions also are important.

The concept of limitation has been broadly defined. An environmental factor can be limiting in a true ecological sense, i.e., a factor limiting the growth or standing crop below its potential maximum growth rate or biomass. Mechanisms of physiological acclimation that optimize growth rates under limiting conditions can be used to develop diagnostic tools. We use the term "diagnostics" to denote a signal or procedure which provides specific information to symptomatically identify an environmental constraint. The aim of diagnostics is to interrogate natural populations without experimentally manipulating them.

Limitation of growth by an environmental factor can become so severe (stress) as to go beyond the tolerance limits of an organism, so causing the replacement or disappearance of a species within an ecosystem. We can stretch the concept of limiting factors to include specific ecological adaptations to a particular environment. These adaptations can be indicative of which factors dominate the dynamics of an ecosystem or a particular niche within an ecosystem. This ecological adaptation can be reflected as a physiological response to a stress (either reversible or irreversible; e.g., adaptation to high pressure; facultative vs. obligate anaerobes).

Selected specific examples of ecologically relevant, rate-limiting processes that can be, or have been, approached with molecular biological techniques are presented below (see also Falkowski and LaRoche, 1991). The potential limiting factors discussed are CO_2/water, nitrogen, iron, trace metals, temperature,

light, and pressure.

CO_2 Limitation/Water Limitation

Photosynthetic organisms play a major role in the global carbon cycle by depleting the atmospheric pool of CO_2. For photosynthetic carbon fixation to reduce atmospheric CO_2, the external factor(s) which limit photosynthetic processes must be alleviated. On geological time scales, however, global photosynthetic processes reach a relative steady-state with respiratory processes. An understanding of the factors limiting primary production is essential to understanding the transient response to anthropogenic CO_2 emissions.

In the terrestrial environment, the major factors limiting biomass production are CO_2 concentrations, water and nutrient availability, fluctuating salinity levels, and temperature. The global distribution of terrestrial plants is related to the availability of water more than to any other single factor. In fact, all three factors limit the availability of water for physiological reactions. Operationally, CO_2 levels, drought, salinity, and temperature are signals that elicit specific responses; only the persistence of the signal causes stress.

One aspect of study must be the pathways by which these signals are sensed and transmitted, what the transmitters are, and how they interact with receptors and target sites to elicit specific physiological and molecular responses. In plants, such stresses involve the hormone, ABA. In all plants studied, these stresses induced the expression of "lea" proteins (late embryogenesis abundant in seeds) in vegetative tissues. The proteins (and their mRNAs) are markers of water stress.

Ribulose bisphosphate carboxylase (Rubisco) is a relatively inefficient enzyme for CO_2 fixation. For example, many cyanobacteria have developed a CO_2 concentration mechanism, whose elucidation was critically dependent on molecular technology (Price and Badger, 1989). Its presence can be inferred from a few proteins for which there are antibody probes. The number of different cyanobacteria species with functional carboxysomes should be an indicator that CO_2 is a limiting factor in marine systems which may seasonally dominate C-fluxes at some latitudes.

Another mechanism of CO_2 concentration is associated with C_4 and Crassulacean-acid metabolism (CAM) plants. These pathways reduce water loss via transpiration per amount of CO_2 fixed. In plants of both groups, CO_2 is intermittently fixed by phosphoenolpyruvate carboxylase (PEP) into malate. In C_4 plants, malate is transported to a specialized tissue, decarboxylated, and the CO_2 is assimilated by Rubisco. In CAM plants, initial fixation occurs at night, when there is little evaporative water loss through open stomatosis flow; final assimilation occurs in the same cells during the day, when energy is not limited, with stomates closed.

The manifestation of both pathways is developmentally programmed and, often, environmentally enhanced. Developmentally and environmentally programmed sensors, transducers, signalling molecules, and their target sites in membranes and in gene control elements are poorly understood. Several gene probes are available, and several mechanistic approaches have been initiated. Induced genes appear to be characterized by cis-acting elements in their promoters, which are recognized by several transacting factors. The elements may confer stress-inducibility, and the factors may be ubiquitous, so it is necessary to assume (an)other control mechanism(s) that act on the protein factor bound to a particular cis-element.

One of the most significant model systems for these studies is the induction of water-efficient CAM metabolism in the C_3 succulent Mesemsynanthemum crystallinum by salt and water stress (Winter, 1985). The model proving most amenable to molecular evaluation of the primary stress-protein response and the subsequent induction of the whole suite of additional metabolic reactions used in the energetically destructive CAM pathway (Bohnert et al., 1988), including the primary CO_2-fixing enzyme PEP carboxylase.

Nitrogen Limitation

The low concentration of dissolved inorganic nitrogen observed in the euphotic zone of large portions of the oceans has prompted biological oceanographers to hypothesize that phytoplankton growth could often be limited by the availability of nitrogen. While the physiological response to nitrogen limitation is well characterized in marine phytoplankton, the molecular basis of nitrogen limitation

on carbon fixation remains obscure. Work on Chlamydomonas reinhardtii grown under nitrogen-limitation showed that all the components for light-energy transduction become severely depleted. In addition, modifications of some of the complexes could be discerned, including the appearance of novel, light-harvesting pigment-protein complexes and a reduction in the amount of chlorophyll bound to the Photosystem I reaction center. While these specific phenomena are not observed in the marine phytoplankter, Isochrysis galbana, losses of peripheral antenna protein complexes occur in response to a reduction in nitrogen supply.

To identify the molecular basis for adaptation to poor growth conditions, rates of photosynthetic protein synthesis and levels of messenger RNAs for photosynthetic proteins were examined. These analyses demonstrated that nitrogen deficiency changes the expression of nuclear genes for pigment-binding proteins: certain normally high, abundant mRNAs disappear while new mRNAs for related, but functionally distinct, light-harvesting proteins accumulate. Within chloroplasts, all mRNAs for photosynthetic proteins are present at normal levels, but only a few are used to control rates of protein synthesis. Together, these data indicate that the availability of nitrogen can influence nuclear gene transcription and translation of chloroplast mRNA. Furthermore, the nitrogen-deficiency phenotype is rapidly reversed when nutrients are added to the cultures.

It is well established that nitrogen status determines the stability of the photosynthetic apparatus and its response to light-dependent damage (photoinhibition) (Ferrar and Osmond, 1986; Henley et al., 1991). A key element in photoinhibitory damage centers on the D-1 protein of the rapidly turning-over PS II reaction center (Kyle, 1987) and on protective mechanisms associated with the xanthophyll cycle (Deming-Adams, 1990). Probing these specific reactions by molecular methods may help describe large system functions (e.g., marine phytoplankton) in global carbon fluxes.

Much remains to be learned about nitrogen deficiency at the molecular, cellular, and ecosystem levels. Are the genes encoding the proteins in the nucleus, or, as DNA sequence data suggest, do some genes reside in chloroplasts? How do cells detect nitrogen-deficiency, and how does it prompt changes in the expression of

genes for chlororespiration and photosynthetic proteins? Is chlororespiration truly necessary for survival of photosynthetic organisms in nitrogen-poor environments? Does chlororespiration develop in all nutrient-deficient photosynthetic organisms, or are there alternative strategies for survival?

Iron Limitation

Some regions of the oceans have high levels of dissolved inorganic phosphorus and nitrogen, and it has been hypothesized that iron may limit primary productivity there. As a result, laboratory studies have been initiated to promote understanding of iron-limitation at the molecular level.

Cyanobacteria can live in environments that differ widely in the amount of available iron. When iron is limited, the cells grow well, but the biomass is lowered and cellular pigmentation is substantially altered, as is the case for nitrogen limitation. Phycobilisomes are no longer formed and the chlorophyll-protein composition is changed. These alterations can be visualized by changes in the absorption spectrum and in the fluorescence spectrum. Among other events is synthesis of a new chl-protein (CP) complex, CP43', which is similar to a normal PSII CP complex, CP43, except that it lacks a 100 amino-acid domain localized in the membrane lumen. This modified protein is synthesized only under iron efficiency and can be diagnostic of this condition. The gene is controlled by an iron-regulated promoter, which appears to be recognized specifically when iron is deficient. A similar phenomenon is found in the marine diatom, Phaedactylum tricornutum.

This system provides a wealth of probes that can be used to analyze iron deficiency in natural populations, including spectral analyses, (a) absorption spectra -- loss of absorption at 625 nm (phycocyanin) and Chl shift to 675 nm, and (b) 77^{0}K fluorescence spectra -- > total loss of 696 nm and 716 nm fluorescence, and a large increase in 685 nm fluorescence (due to new CP complex). The antibody against a portion of the new protein (CP43') is highly specific and can be used as a probe for detecting iron limitation. A DNA probe can detect the expression of gene under iron-deficient conditions. A promoter can be cloned in front of the reporter gene, whose expression will be turned on only under iron deficiency. If a genetic transformation system is available, such

constraints can be incorporated into cells and used as an *in vivo* indicator of iron deficiency.

Reporter genes have been used to detect *in situ* activity of microbes a few times. New developments in molecular genetics allow us to measure the transcriptional activity of individual genes *in situ*. While the product of many environmentally important genes may be difficult to assess directly or indirectly *in situ* due to their low level of expression, such genes can be fused with genes whose products are easily measured *in situ*. The promoterless "reporter gene" will be expressed only when the environmental gene is transcriptionally active. A promoterless, ice- nucleation gene was recently shown to be a sensitive reporter of the transcriptional activity of iron-regulated *Pseudomonas* genes that are involved in siderophore-mediated iron uptake *in situ* in soil. Soil does not contain ice nuclei, and the transcriptional activity of the iron-sensing gene in root-colonizing *Pseudomonas stomis* could be easily quantified in a simple, droplet-freezing assay that measures the number of ice nuclei produced by containing the reporter-gene strains introduced into the soil. These studies showed the average level of Fe^{3+} sensed by bacteria on roots, and demonstrated the heterogeneity of this resource *in situ*. Such an approach can be immediately applied to all culturable organisms which can be genetically manipulated. Many resource-responsive genes already have been identified that can be fused with appropriate reporter genes to indicate what levels of these resources are sensed by the organism *in situ*. Organisms containing reporter genes can be introduced into different habitats and used as sensitive "biological sensors" of the transcriptional status of other pertinent genes. Further advances in the ability to genetically manipulate many microbes will be needed, but the benefits from using such genetic tools should justify this effort.

Tolerance and Sensitivity to Trace Metals

Toxic trace metals from several different industrial and energetic processes contaminate soil and water. The ability of plants to grow in soils containing normally toxic concentrations of these ions has been known for some time, but the molecular and biochemical mechanisms of metal tolerance are not well understood. Oceanic phytoplankton are extremely sensitive to certain trace metals, while coastal species are relatively tolerant.

To understand the molecular factors underlying these mechanisms in higher plants, cultures of plant cells were established from metal-tolerant and -sensitive plants. Analysis of the cells demonstrated that part of the mechanism of tolerance involves the de novo synthesis of glutathione-derived, metal-binding polypeptides that have high binding affinities for Cd, Cu, Zn, Hg, and other metal ions. In metal-tolerant cells, these peptides are normally undetectable. However, de novo synthesis can be detected within 5 minutes of exposure to metal ions, and these peptides reach 6% of the total protein in the cells within 12 hours.

Polypeptide synthesis results from the catalysis of a reaction by the enzyme gamma-glutamylcysteinyl dipeptidyl transpeptidase which consumes glutathione. This enzyme is constitutively present in all plant tissues, and is directly activated by the binding of Cd or Cu (and, perhaps, other ions) directly to the enzyme.

At least 34 different genes are up- or down-regulated or differentially expressed in tolerant vs. sensitive cells (and plants) upon exposure to metal ions. Some genes are specific to Cd or Cu exposure, but others also are expressed in response to other stresses. In addition, seven enzymes are involved in the biosynthesis of metal-binding polypeptides and their precursors. Each enzyme is encoded by one or more genes, which may be directly or indirectly regulated by metal exposure.

Preliminary experiments demonstrate that DNA probes for such genes can be used to determine whether or not they are active in plants growing in the environment. The presence of a metal-induced gene product (mRNA) may, therefore, indicate the presence of the metal ion in the soil. Moreover, because the metal ion must be taken up by the plant for expression to occur, information is provided about the mobility of the metal ion within the biosphere.

Temperature Limitation

Temperature is a fundamental environmental factor which geographically controls many ecologically important activities and ranges of species. Sudden or gradual temperature changes, or extreme temperature perturbations can result in new or modified activity, reflecting either temperature-induced changes in metabolism or altered gene expression.

A. Freezing Tolerance and Low Temperature-induced Genes in Higher Plants

A specific example of how temperature can affect species distribution is provided by plants of temperate regions. A unique feature of most temperate plants is their ability to survive by developing freezing tolerance in the fall and winter in response to the shortened photoperiod and colder temperatures.

Most hardy plants develop freezing tolerance when exposed to temperatures between 0-10^{0}C in controlled environments. Since 1970, it has been proposed that cold acclimation involves the alteration of gene expression. Because a series of cellular changes occur during the development of cold hardiness, it is thought that specific enzymes might be induced. Molecular approaches amply support this notion; specifically, several "cold inducible" genes have been isolated. Northern blot analysis and nuclear run-on experiments demonstrated that those genes are regulated at the transcriptional level.

Many temperate plants can detect changes in temperature, and translate the signal into the expression of specific genes, which, in turn, confer freezing tolerance to the cells. Currently, the function of those genes is not known. Future research will characterize these genes, analyze their promoters, and elucidate the function of the gene products.

B. Bleaching of Corals

Approximately 30% of the shallow sea floor to 30m in the tropics (i.e., between 30^{0}N latitude and 30^{0}S latitude) is dominated by reef-building corals, and coral reef communities. The stability of these communities worldwide is linked to the sustained residence in the coral's cells of symbiotic dinoflagellates (zooxanthellae). When the temperature is increased to a few degrees above ambient (to 32^{0}C), or to 10^{0}C below ambient (to 15^{0}C), the corals release their zooxanthellae. The result is diminished calcification, loss of the products of zooxanthellae photosynthesis, and death of the corals.

How does the change in temperature destabilize the coral-dinoflagellate symbiosis, evoke release of zooxanthellae, and

decrease calcification? One approach to understanding the basic mechanism is to determine the molecular basis of bleaching, i.e., loss of chlorophyll, that precedes or is concomitant with the loss of symbiotic algae. A model approach can be drawn from the work of Ortiz (1990) and Ortiz et al. (1991) on "heat bleaching" in Euglena.

The alga Euglena gracilis can be grown indefinitely at 30°C. However, after a few generations at 33°C, cells are found that contain rudimentary plastids and are permanently bleached; this phenomenon has been linked to a progressive failure in the translational activities of chloroplasts (Ortiz, 1990). The earliest event detected is the abrupt disappearance of normal transcripts probed by psbE, which codes for one of the subunits of cytochrome b_{559} and is one of a cistron of six genes (Price, G.D., pers. comm.). In the present case, a candidate for such a process is a switch from one transcriptional start site to another. A second is a change in the processing, e.g., splicing of transcripts.

Light Limitation

Light limits photosynthesis in many marine and terrestrial environments. Models of succession in higher plant ecosystems are driven by competition for light between species. In coastal ecosystems, light limitation is the primary factor controlling the rate of carbon fixation by phytoplankton.

All photosynthetic organisms have some ability to adjust the absorption cross-section of the photosynthetic apparatus in response to changes in irradiance (Falkowski and LaRoche, 1991). In phytoplankton, for example, the absorption cross-section may increase by a factor of three or more as cells are grown at lower irradiance levels, thus increasing light-harvesting ability (Falkowski and LaRoche, 1991). Higher plants have a much reduced capacity to photoacclimate (Anderson and Osmond, 1987).

The molecular basis for photoacclimation is poorly understood. In the marine green alga, Dunaliella tertiolecta, the abundance of transcript for the light-harvesting chlorophyll proteins increases five-fold within nine hours following a shift to lower irradiance levels (LaRoche et al., in press). Subsequently, the transcripts

are translated, leading to an increase in antenna size. However, if chlorophyll biosynthesis is blocked at an early stage, or cells are placed in the dark, the increased transcript level is not expressed as an increase in the pigment protein complex. The preliminary work on this model system suggests that the transduction signal is processed at the transcriptional level (LaRoche et al., in press). The nature and mechanisms of the controls remain unclear, as does the significance for the reduction of the process in higher plants. Comparative analysis will lead to an understanding of how light intensity signals are perceived and transduced in photosynthetic organisms.

Pressure Limitation

Approximately 80% of the ocean's volume is below 1000 m in depth, making the high pressures encountered in this habitat the most prevalent in the biosphere. Pressure strongly delineates and limits the distribution of oceanic life, with an influence as significant as that of temperature, salinity, or currents (Yayanos, 1986). Deep-sea organisms, which have evolved specific biochemical and physiological adaptations to deal with hydrostatic pressure, may play a role in the processing and recycling of biogeochemically important compounds (Longhurst and Harrison, 1988). Additionally, a variety of marine organisms undertake diurnal or ontogenetic migrations between the deep sea and surface waters where they can influence the population structure of surface ecosystems (Ainley et al., 1986; Wakefield and Smith, 1990). The effect of pressure also is important for deep subsurface microbial communities.

Compared with other physical signals such as light and temperature, relatively little is known about how organisms perceive and respond to changes in hydrostatic pressure. Pressure acclimation is believed to involve alterations in many cellular processes, and appears to be manifested at the level of protein and membrane structure and function. For example, in deep-sea organisms the essential properties of enzyme function, such as ligand binding, catalytic rate, and structural stability, are maintained under pressures which severely perturb these features in non-adapted enzymes from surface-dwelling organisms (Siebenaller and Somero, 1989). With increasing environmental pressure, the membranes of bacteria and fishes display an increase in the ratio of total unsaturated to total saturated fatty acids, which has been interpreted as a homeoviscous adaptation to maintain membrane

fluidity (DeLong and Yayanos, 1985; Cossins and Macdonald, 1986). Deep-sea eubacteria and archaebacteria also elevate the steady-state production of specific proteins as a function of growth pressure in a barometer-like fashion. In one abyssal bacterium which has been studied in detail, a gene encoding an outer membrane protein is transcriptionally regulated by elevated hydrostatic pressure (Bartlett et al., 1989). This protein appears to function as a generalized diffusion channel for nutrient uptake.

The structure-function rules which govern the molecular changes conferring pressure adaptation in proteins have not been determined. Future research will focus on the processes by which deep-sea organisms sense and respond to variations in pressure, the regulation of specific genes by pressure, and the function and conservation of those gene products which are essential for pressure adaptation.

Appendix II
Molecular Approaches to Understanding Energetics

Molecular approaches to understanding the inherent limitations of metabolic processes require measurements of internal fluxes. Molecular techniques for measuring fluxes are difficult to apply in natural systems; however, some areas of research are promising.

Molecular Methods for Estimating Growth

RNA/DNA ratios have been used (with mixed success) to estimate bulk growth rates of microbes *in situ*. Can these measurements be coupled to single-cell analysis by use of fluorescent probes for DNA and RNA? These, in turn, can be detected and quantified by flow cytometry or by quantitative epifluorescence microscopy.

Fluorescently labelled antibodies to cell-division cycle (CDC) proteins may indicate dormant vs. active stages. Can such techniques be used to calculate growth rates, using algorithms similar to those used in frequency of dividing cell (or frequency of DNA replication) methods for growth-rate determinations (Carpenter and Chang, 1988)?

If these life-cycle stages are metabolic stages, i.e., dormant vs. active, photoautotrophic vs. mixotrophic, they may be characterized by different rates and/or by different pathways. If there are different pathways, are they characterized by different products that can be used as biomarkers to identify those life-cycle stages *in situ*? If these stages and their associated pathways are also correlated with different rate functions, we may be able to deduce rates by presence of the molecular biomarkers.

Flow cytometry has been used to enumerate bacteria in aquatic systems. The technique would be useful in looking for an organism that comprises less than 1% of the population. The drawback to flow cytometry is that it fragments consortia. However, if the proper methods are found, particulate matter containing bacteria or intact communities possibly could be sorted to obtain much the same information as described above.

Evidence from the response of starved *E. coli* (Matin et al.,

1989), marine Vibrio species (Nystrom et al., 1990), and other gram-negative bacteria (Grimes et al., 1986) suggests that the identification of growth states might provide an alternative to measurement of growth rate. Organisms exposed to the stress of severe starvation exhibit so many new proteins that their adaptation is reminiscent of sporulation; that is, bacteria may have a complex developmental response to nutrient limitation involving more than one state. This developmental process results in a dormant form of the organism that is more energy-efficient and less susceptible to heat and other environmental insults (Chesbro et al., 1990; Morita, 1985). Thus, the growth state of an organism in a natural population may provide clues not only to the nature of the stress (carbon, nitrogen, or iron limitation), but also to the extent and duration of the stress.

Identifying Dominant Metabolic Pathways in an Organism

The importance of identifying the dominant metabolic pathways can be seen from two examples:

Many phytoplankton in the chrysophyte/prymnesiophyte line live in close association with their bacteria and cannot be maintained in axenic culture. The nature of the association is not yet clear. In some cases, the bacteria may be vitamin sources; in others they are alternate carbon sources. The method of ingestion is phagotrophy; that is, the phytoplankton ingest (and presumably digest) whole bacteria. While at first glance this life style may seem inefficient, in a fluctuating environment (delta light and nutrients), it makes good sense. The phytoplankton photosynthesize when conditions are optimal, but rely on phagotrophy when they are not, with symbiosis optimizing DOC transfer or respiration back to CO_2. Thus, the unit of selection (unit of persistence in the environment) is the association; similarly, with regard to fluxes of energy and carbon, the unit again is the association and not the individual.

Possibly there is a metabolic switch from photoautotrophy to phagotrophy, analogous to a switch from the active to the dormant stage. Here also, we need to identify pathways and products that would provide biomarkers. A comprehensive integrative method for large-scale studies depends on multiple stable isotope techniques to trace autotrophic and heterotrophic inputs with natural

abundance ^{13}C and ^{2}H ratios. Genetic transformation of Chlamydomonas (Boynton et al., 1990) is being used to evaluate the molecular bases for changes in isotopic fractionation (Osmond et al., 1991).

Energetics of Global Carbon Cycling

Two geochemical indicators of the biospheric role in the global carbon cycle are the following:

a. The annual variation in the northern hemisphere of $^{13}C/^{12}C$ in atmospheric CO_2, consistent with participation of Rubisco in the draw down of CO_2; and

b. The annual variation in the northern hemisphere of $^{18}O/^{16}O$ in atmospheric CO_2, consistent with exposure of the entire atmospheric CO_2 pool to carbonic anhydrase in leaf water, which can be a much larger effect than that due to cloud-water equilibration or ocean mixing.

Tans showed that in 1979 the northern hemispheric terrestrial biosphere was the largest contributor to net flux of CO_2 via (a), but in 1989 the southern ocean biosphere was the dominant path for the net flux (Tans et al., 1990). Yakir showed, for example, that the delta O-18 of metabolic water, and not transpiring water, was probably the source of (b). Further research in both areas could underpin fundamental issues in the global carbon cycle. This research can be facilitated by the following molecular techniques (Yakir et al., 1991).

(1) Specificity factor of Rubisco.

Rubisco, the primary carbon assimilating enzyme, is found in several of forms which are diagnostic for groups of organisms. The most common hexadecameric (L_8S_8) form has evolved to perform carboxylation/oxygenation at different rates and efficiencies. While the amount of mRNA for LSU and SSU subunits probably has no indicative value, it is possible that peptide antibodies against specific functional domains or protein modules can be designed. However, the lack of knowledge, especially about chrysophyte, rhodo-, phaeo-, Rubisco enzymes, prohibits such generalizations. In addition, even among cyanobacteria and photosynthetic bacteria,

only a few enzymes have been characterized.

Variations in subunit sequence and structure are only one parameter that might be used for diagnosis. A reflection of structure is the strength of L/S binding. Microcolorimetry, using purified enzymes, could be used to measure this parameter. Along with tight-binding of subunits, inhibitor binding and "misfiring" of the enzyme should be of value. Bound inhibitors (e.g., CABP and CA1P) and misfired products (e.g., xylulose bisphosphate) could be measured by HPLC after destroying the enzyme. Although much is known about the enzyme, little is known about its structural and biochemical features in most organisms; apart from higher plants (where probably a couple of hundred Rubisco enzymes are characterized), only one or two model species are studied in other groups. Yet another aspect of the problem is the activation of inactive enzymes by Rubisco activase. We need to know more about the biochemistry of activation, the activase isogenes, and isoforms of this enzyme.

One of the most relevant parameters in understanding the regulation of Rubisco activity is the so-called specificity factor; namely, the preference of the enzyme to accept CO_2 over O_2 as the substrate. The ratio of specificity of oxygenase and carboxylase activities for Rubisco has been evaluated in only a narrow range of organisms, few of them relevant to the bulk of CO_2 flux through the biosphere. This flux will be sensitive to the specificity factor. The specificity factor, τ, has changed during evolution from 10 (photosynthetic bacteria), to 50 (cyanobacteria), to 70-100 (higher plants). Without a suitable site-directed mutagenesis system in which to study how the primary structure of the enzyme is related to the specificity factor, comparative molecular biological approaches are needed. Adaptations of Rubisco to ecological niches appear likely, and the study of unique eukaryotic systems, which have different catalytic subunit sequences, provides a basis for such an approach.

Surveys of sequence variation are needed, coupled with measurements using mass spectrometry (simultaneous O_2 uptake and CO_2 evolution), in a wide range of relevant terrestrial and marine organisms.

(2) Participation of carbonic anhydrase in CO_2 <---> H_2O

equilibration.

The importance of the intracellular location of carbonic anhydrase in prokaryotic autotrophs to their ability to take up and concentrate CO_2 for photosynthesis has been established recently by the controlled expression of the foreign gene in these organisms. The CO_2 concentration mechanisms in these organisms and other phytoplankton are essential to their role in global carbon flux.

More research is needed on:

(1) the CO_2 concentrating mechanisms of prokaryotic/eukaryotic phytoplankton;
(2) the role of carbonic anhydrase in these mechanisms; and
(3) the role of extracellular carbonic anhydrase and excreted active carbonic anhydrase in facilitating CO_2 <---> HCO_3 <---> H_2O exchange in the ocean.

This research is ripe for transformation technology, and probing of/screening for carbonic anhydrase at the process/physiological level, both supported by mass spectrometry.

(4) Analysis of photosynthetic respiratory activities of mixed populations of phytoplankton using fluorescence quenching and analyses of stable isotope exchange.

All of these research areas are of basic importance in understanding ecosystem energy balance, whether it be concerned with balance of CO_2 fluxes, residence time of carbon, or seasonal and annual trends in greenhouse CO_2.

Sequestration of Carbon: Suggested Techniques and Approaches

There is a need to examine carbon isotopic discrimination of the C-fixation enzymes and enzyme systems in autotrophs: specifically, isotope ratios in specific cellular and subcellular components:

a. Examination of humics, tannins, and "black" muds with gentle extraction and tagging procedures, coupled with chemical analysis. Coupled with separation techniques and high resolution isotope ratio mass spectrometry, this approach could yield valuable

insights into present and past patterns of carbon fixation and cycling.

b. Fluxes: Isotopic methods could be used to study net fixation by using $H^{13}CO_3$ at levels just above background in large enclosure studies in lakes and oceans. Such studies could reveal the ratio of carbon fixed to that incorporated into various primary producers, and add insight into the types of carbon compounds most useful as indicators of primary productivity, recycling of carbon, and burial of carbon.

c. Fates: Not only are the isotopic methods discussed above relevant to the fates and burial of carbon (i.e., for estimating the activity of reprocessing by $^{13}C/^{12}C$), but many modern techniques of molecular genetics are also relevant. For instance:

i) reprocessing of carbon can be estimated by carbon isotopic analyses;

ii) the existence, abundance, and activities of organisms active in diagenetic cycling of carbon; and

iii) indications of microbes and enzymes present in the environment, by using nucleic acid probes and immunological methods.

Coupling of type (b) studies with those discussed in (a) may provide valuable insights into local and global carbon cycling. For example, within deep marine or lacustrine muds, species could be identified which have disproportionately contributed to organic carbon sedimentation, by examining residual DNA fragments. The contribution of broader taxonomic groups may be identifiable from ratios of stable isotopes (e.g., $^{12}C:^{13}C$ of carbon compounds, such as fatty acid and lipid skeletons, and residues). The ratio of identifiable to amorphous material may provide insight into the decay processes that precede sequestration. Various isotopic dating methods of depth-graduated samples can estimate carbon flux to carbon sequestered. Similar methods apply to the study of terrestrial soils.

Calcification

In the oceans, photosynthetic CO_2 fixation reduces the partial pressure of CO_2, thereby increasing the CO_2 diffusion gradient between the atmosphere and the ocean. In contrast, calcification

(deposition of $CaCO_3$) counterbalances this trend by reducing the buffering capacity of the upper oceans, and increasing the partial pressure of dissolved CO_2. All of the $CaCO_3$ in the earth's crust has been deposited as a result of biological activity. The $CaCO_3$ reservoir is the largest pool of non-aqueous carbon on earth. Curiously, despite the importance of calcification in the deposition of carbon on geological time scales, almost nothing is known about the molecular basis of the process.

Three major groups of oceanic planktonic organisms, foraminiferans, coccolithophores, and pteropods, secrete a calcium carbonate skeleton. Among the benthos, certain areas of the oceans are dominated by organisms that deposit calcium carbonate. For example, approximately 15% of the shallow sea floor between 0 and 30 m is occupied by coral reef communities whose primary productivity and calcification contribute significantly to global carbon flux. Unexpectedly, coral reefs from different latitudes and with different biological components exhibit considerable physiological uniformity. Their photosynthesis:respiration ratio is about 1.15, and their rate of calcium carbonate deposition is about 4 kg $CaCO_3$ m^{-2} y^{-1}. This apparently "standard rate" provides a quantitative basis for checking the effects of large-scale environmental stress (i.e., at the community level). In fact, nutrient overload (e.g., elevated nitrogen and phosphorus) changes the standard rate of community metabolism which can be measured long before any changes in community composition can be detected visually.

Organic matrix proteins secreted by calcifying tissues are thought to control both calcium carbonate nucleation and species-specific skeletal architecture. Molecular biological approaches may provide insight into the synthesis of matrix proteins, many of which are beta sheets rich in aspartic acid residues: they may be thought of as calcium carbonate synthetases.

Appendix III
Molecular Approaches to Understanding Community Structure and Function

Phylogenetic-based Probes to Determine Community Composition and the Abundance and Distribution of Organisms

Baseline data on community structure and variability are prerequisite for understanding the effects of environmental perturbation on species diversity and community structure and function. Detailed analyses of community structure, diversity, and variability have been largely limited to ecosystems which are spatially and temporally readily described and relatively stable. Terrestrial forest ecosystems, for example, with discrete boundaries and readily identified species, have been extensively characterized by systems ecologists. Our ability to assess the diversity and structure of ecosystems with less easily identified species, extremely small spatial scales, or ill-defined boundaries has been hampered by lack of appropriate methods. To assess the impact of environmental perturbation on ecosystem structure, it is critical to have baseline information on species abundance and variability for which facile identification and quantitation of individual species is necessary. For bacteria, protozoa, and the adult and developmental stages of many metazoans, even reliable species identification may prove difficult. Molecular techniques can resolve some of these difficulties.

Acquisition of detailed information about community structure, function, and diversity has been hampered by a lack of appropriate methods. In particular, the identification of microorganisms in natural samples generally requires axenic cultivation of the resident organisms, yet we can culture less than 1% of the microorganisms in most environments. Additionally, varying efficiencies in culturing different organisms introduce uncertainties in the enumeration of microorganisms in environmental samples. To address these difficulties, molecular methods are being developed, using gene sequences, in particular of ribosomal RNA (rRNA), to identify and quantitate microorganisms in natural samples without the need for cultivation.

If the rRNA sequence of an uncultivated organism can be determined, the evolutionary relatedness of the organism to known

ones can be established using techniques of molecular phylogeny (Olsen, 1988). By establishing phylogenetic relationships, some properties of an otherwise unknown organism then can be predicted because representatives of particular phylogenetic groups are expected to have common properties. The same accumulated mutations that are the basis of the phylogenetic analysis provide sequence variability that can be used to identify and quantify organisms in the environment by hybridization with organism-specific probes.

The phylogenetic identification of organisms can be determined without cultivating them by extracting total nucleic acids from a sample and analyzing rRNA sequences, as shown in Figure 1. The four approaches outlined include (i) direct sequencing of purified 5S rRNAs; (ii) sequencing from a cDNA library of rDNAs following reverse transcription of extracted RNAs; (iii) cloning and sequencing of PCR amplified rRNAs; or (iv) cloning size-fractionated DNA into phage lambda, and purifying and sequencing cloned rDNAs. rRNA sequences are compared to a data base of known rRNA sequences to identify phylogenetic relationships.

Conserved and variable regions of rRNA sequences are useful as hybridization sites for synthetic oligonucleotide probes to identify or to distinguish organisms. The rRNAs are particularly attractive hybridization targets, because there are as many as 10^4 to 10^5 ribosomes per cell. In situ hybridizations between isotopically or fluorescently labeled probes and formaldehyde-fixed cells permit the phylogenetic identification of individual cells (Giovannoni et al., 1990; DeLong et al., 1989). One set of fluorescent probes developed for single-cell analysis consists of oligodeoxynucleotides that distinguish the three primary lines of evolutionary descent: eubacteria, eukaryotes, and archaebacteria (DeLong et al., 1989). Epifluorescence microscopy shows that a eukaryotic-specific probe hybridizes selectively to cells of Saccharomyces cerevisiae in a mixture of S. cerevisiae and Bacillus megaterium, while a fluorescently labeled probe complementary to a universally conserved region of the rRNA anneals to both. Similar fluorescent probes also have been used to examine the microbial ecology of mouse cecum samples (Amman et al., 1990).

The combination of these techniques provides the potential to identify microorganisms, visualize single cells, and quantitatively assess the abundance of specific microorganisms, all without

cultivating the organisms.

Three microbial assemblages have been characterized using 5S rRNA sequences: the bacterial symbionts of invertebrates that surround deep-sea hydrothermal vents (Stahl et al., 1984), the bacteria inhabiting the 91^{0}C source pool of Octopus Springs in Yellowstone National Park, Wyoming (Stahl et al., 1985), and the microorganisms inhabiting a copper leaching pond

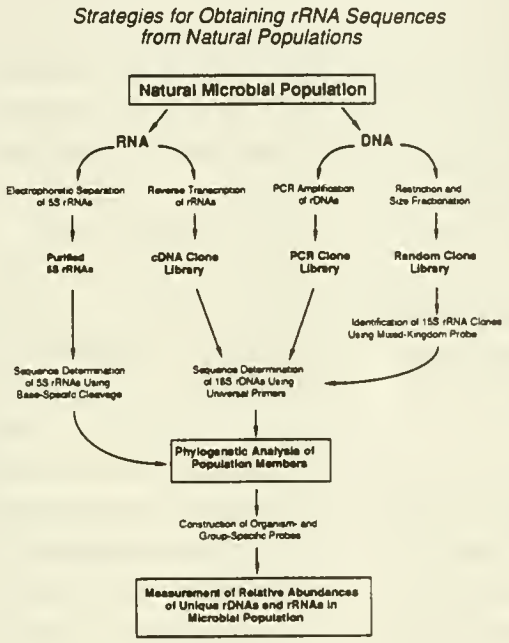
Strategies for Obtaining rRNA Sequences from Natural Populations
Natural Microbial Population
RNA
DNA
Electrophoretic Separation of 5S rRNAs
Reverse Transcription of rRNAs
PCR Amplification of rDNAs
Restriction and Size Fractionation
Purified 5S rRNAs
cDNA Clone Library
PCR Clone Library
Random Clone Library
Identification of 16S rRNA Clones Using Broad-Kingdom Probe
Sequence Determination of 5S rRNAs Using Base-Specific Cleavage
Sequence Determination of 16S rDNAs Using Universal Primers
Phylogenetic Analysis of Population Members
Construction of Organism- and Group-Specific Probes
Measurement of Relative Abundances of Unique rDNAs and rRNAs in Microbial Population

Figure 1.

at the Chino Mine in New Mexico (Lane et al., 1985). The resolution of a phylogenetic analysis using the relatively small 5S rRNA is considerably less than that possible with the greater number of sequence position available in the 16S or 23S rRNAs (Pace et al., 1986).

The cDNA approach was used to examine a microbial mat in Yellowstone Park (Weller and Ward, 1989; Ward et al., 1990) that was previously studied by isolating organisms from the mat (see Brock, 1978). The molecular approach confirmed earlier suspicions that many members of the mat community had not been cultured in the laboratory.

Efforts are under way to identify the composition of marine picoplankton collected from oligotrophic oceanic waters using

ribosomal RNA sequences. Giovannoni et al. (1988) used universal rRNA primers and the polymerase chain reaction (PCR) (Saiki et al., 1988) to amplify rRNA genes from picoplankton collected in the Sargasso Sea. A preliminary study in the Pacific Ocean using rRNA genes isolated from a picoplankton clone has also been completed (Schmidt et al., 1991). One noteworthy conclusion is that a cluster of closely related bacteria was found in both the Atlantic and Pacific Oceans. Early estimates suggest that this group of bacteria may comprise 10-15% of the marine picoplankton from these areas, and yet the group is not closely related to any cultured organisms for which rRNA sequences are available. If these estimates are correct, understanding their distribution and metabolic capacity will be crucial to an analysis of the community.

Molecular Assay of Intraspecies Variability

Elucidation of the extent and role of intraspecies variability is essential to understand how environmental stress or perturbation may influence community structure and function. Intraspecies variation may be an important buffer, maintaining community stability in the face of environmental challenge. Molecular data from bacterioplankton in the Sargasso Sea (Giovannoni et al., 1988) and Central North Pacific imply that there is a large degree of genetic diversity between closely related, coexisting marine cyanobacterial populations. However, the overall extent of intraspecific variability, its function and maintenance, are little understood in these and other environments. For instance, it was recently shown that viral populations are extremely abundant in the oceans, present in about the same order of magnitude as bacterioplankton populations ($\approx 10^6$/mL; Suttle et al., 1990). These populations are thought to be important in controlling the size of phototrophic and heterotrophic microbial populations, and hence, they may greatly influence both primary and secondary productivity. Yet what prevents these presumably lytic viruses from eliminating their host populations? Is intraspecific variation an important factor in determining host-viral interactions? Can environmental perturbance influence the interactions between viral and host populations, or skew the intraspecific variability which may buffer these populations? The answers to these and similar questions will provide important information on the influence of chronic stress on intraspecific variation, and its consequences.

A host of techniques to detect molecular variants are available to assay intraspecific variability. For example, protein polymorphisms detected by isozyme electrophoresis have been used to describe allele frequency changes in time and space. Immunological techniques can detect intraspecies antigenic variation at the level of the single cell. The polymerase chain reaction will be particularly useful to isolate and detect variants at the genetic level, whether in the mitochondrial, chloroplastic, or nuclear genomes.

The genomes of eukaryotic organisms contain many repetitive DNA sequences. In some cases (e.g., the human "Alu" sequence), specific repetitive sequences are distributed throughout the genome; in other cases, a specific repetitive sequence may be confined to one or few loci. Chromosome-specific sequences in humans have been used to identify and quantify specific chromosomes from mixed populations. The "Alu" sequence has been used to differentiate among human, hamster, and human-hamster hybrid chromosomes.

The same technique could be applied to identify and quantify specific species of microbes within a population. The presence of sequence repeats increases the sensitivity of the method; therefore, expression of the gene is not necessary for success. Moreover, there is no need to understand the function of the sequence. Such sequence probes could, therefore, be isolated from mixed populations of organisms, sequence specificity determined, and the species containing a specific repetitive sequence identified. Once this basic characterization is completed, the repetitive DNA probe could be used to identify the presence and number of the species within a larger, mixed population of organisms.

Molecular Determination of Mutation Rate and Evolutionary Origins

The DNA preserved in some ancient materials is essentially a "molecular fossil," providing a genetic record of the evolutionary past. Molecular techniques, in particular the polymerase chain reaction (PCR), now allow reasonably straightforward access to this "molecular fossil record." Via PCR amplification of ancient DNA, both extinct and contemporary species have been compared using molecular phylogenetic analyses (Paabo et al., 1989). A recent example of this approach examined the historical epidemiology of

the causative agent of Lyme disease. By applying PCR to archival museum specimens of the vector of Lyme disease (ticks), it was possible to retrieve and analyze preserved DNA from preserved samples. This led to the discovery that the historical entry of Lyme disease into the United States occurred before the disease was clinically recognized (Persing et al., 1990). Such techniques applied to older samples may allow molecular characterization of ancient species, and a better understanding of the processes influencing their evolution. In a similar fashion, can we correlate genetic changes documented in the geological record? Does rapid environmental change correlate with rapid evolutionary change at the genetic level? Such information would provide insight into the genetic responses of individuals and species to environmental fluctuations.

Is environmental stress a significant factor eliciting genetic change, and if so, how? Just as chronic stress may influence population dynamics and species composition, it may also influence the rate (and possibly direction) of genetic change (mutation) in individuals. For example, the classic paradigm of Neodarwinian evolution presumes that the probability of any individual mutation occurring is essentially random, and relatively independent of environmental influences. However, recent evidence indicates that mutations at some genetic loci may, in fact, arise at a greater frequency under specific environmental conditions. Missense mutations (from tryptophan auxotrophy [trp-] to tryptophan prototrophy [trp+]) in tryptophan-requiring strains of E. coli occur at much greater frequency under (nonselective) conditions which are advantageous for resulting mutants, than under neutral conditions (Hall, 1990). Yet mutation rates at other genetic loci do not increase during tryptophan starvation, and starvation for other amino acids does not increase the rate of tryptophan mutations. It appears, then, that at least for the tryptophan operon in E. coli, mutation rates can be specifically increased under certain specific environmental conditions. This is a clear example of how incompletely we understand the environmental factors, and genetic responses, which may influence the rate and direction of mutational change. How prevalent is this phenomenon for other organisms, and at other genetic loci? Do environmental stresses increase mutation rates, and if so, are they great enough to perturb community structure and function? Do anthropogenic environmental changes significantly increase the rate of mutation in natural populations?

Laboratory-based studies of mutation frequencies and distributions will be a useful starting point for understanding the effect of environmental factors on the rates of genetic change. In conjunction with phenotypic analyses, assays of molecular variants, such as restriction fragment length polymorphism (RFLP) analysis and/or PCR random amplified polymorphic DUA(RAPO) (Williams et al., 1990), will be critical. PCR is sensitive enough to detect single point mutations from crude nucleic acid extracts, and analyses are reasonably rapid. A future challenge will be the assessment of natural mutation rates, and how environmental stress might influence these rates. These studies will necessarily be linked to an understanding of the extent and role of intraspecies variability. Identification of model populations will be important for the study of mutational frequency amongst natural populations.

Molecular Probes for Organic Function

Environmental perturbation may profoundly affect the functional roles and interactions between co-occurring species. In some communities, particularly microhabitats, functional interactions are still poorly understood. Microbes do not exist as individuals in the environment, but interact as closely linked, interdependent communities. In some instances, undue stress on one member of a microbial consortium may have a profound effect on the entire community structure and function. In other cases, functional redundancy among different members may buffer environmental stress. Molecular analyses of population structure, such as those described above, have the potential to detect covariance between species, and species interdependence. In addition, molecular probes applied at the <u>in situ</u> level may provide information on the spatial relationships and interactions between species.

The significance of molecular approaches for studying the functions of organisms, and factors which limit function, are described below. The use of molecular probes to assay and detect functional gene and mRNA sequences present in key proteins, linked to species and group-specific molecular probes, may allow correlation of species to function with <u>in situ</u> assays, so allowing simultaneous dissection of communities for both species composition and functional redundancy. Differential expression of related genes in different species could also be detected.

For identifying organisms and populations, the concept of "species" may or may not be useful in predicting succession in the plankton or in the sedimentary environment. The concept of "functional groups" may be more useful. (Often, but not always, functional groups will be taxonomically based. The "species," however, may not be the important functional unit.) A species appears in the plankton and becomes "abundant" in part because it has the necessary pathways or molecular apparatus for survival. In part, this is a stochastic function of whether or not the species is present in sufficient numbers to serve as an inoculum. The classification of "functional" groups is a formidable problem and is primarily a conceptual one, in the sense of defining the set of environmental conditions in which the species must function to persist.

Functional groups based on metabolic pathways and other parameters are discussed below, to emphasize some of the possible routes that might be taken in the solution of this obviously very complex issue.

Determining Organism Function in Consortia

Many geochemical processes co-occur in microbial assemblages such as mats, planktonic and soil consortia. Consortia means a suite of organisms interacting as a functional group within a larger community of organisms. Degradation of a chlorinated hydrocarbon pesticide by a group of microorganisms, each performing part of the overall reaction that destroys the pesticide, is an example of a consortium which can be studied in the laboratory. Many processes in carbon, nitrogen, and sulfur cycles are likely to be mediated by similar consortia. Processing of nitrogen in microbial mats is such an example, in which multiple opposing transformations (e.g., oxidation and reduction of nitrogen oxides) are mediated by diverse but physically and biochemically coordinated organisms. Net chemical fluxes through consortia are determined by the integrated activity of the consortia members and their response to external cues.

Microbial Loop of Marine Food Webs

Some general details are known about the community structure of marine webs; i.e., a large fraction of their carbon is cycled through the "microbial loop." The species composition of large

eukaryotic phytoplankton has been fairly well characterized by classical morphology, and major classes of protozoa have similarly been identified. Epifluorescence microscopy has provided details about the abundance of phototrophic and heterotrophic prokaryotes. Within the oxygenic photosynthetic prokaryotes, two major groups occur in the marine environment (cyanobacteria and prochlorophytes) and several groups are found in freshwater. The identification of heterotrophs, however, is difficult, largely because many cannot be cultured.

Several interactions within the microbial loop are particularly interesting in understanding the stability and structure of microbial communities. Competition is an extremely important process which affects their overall structure. How do variations in resources, such as different nutrients, affect the species composition in aquatic microbial loops? Production is extremely important in the dynamics of microbial loops. How do changes in specific protozoan populations affect the diversity of their prokaryotic prey? Does grazing increase species diversity by removing the dominant prey species? What are the grazing specifications of particular protozoans?

Microbes do not exist as individuals in the environment, but interact as closely linked, interdependent communities. Stable pH and oxygen gradients have been measured in some marine particulates, indicating that anaerobic processes may take place within aggregate microhabitats, in an otherwise aerobic environment. These transient communities may be extremely sensitive to perturbation, which could have a dramatic effect on carbon flow through the system. What is the community structure of the microhabitats, and their sensitivity to physical perturbation? What successional events take place during community change? How do these affect community function?

The function of viruses in microbial communities needs to be assessed. Proctor and Fuhrman (1991) report that viruses in microbial food webs are potentially important in both organic matter release and particle formation. Host defense mechanisms against viruses can include the release of proteases and nucleases. Molecular methods are likely to be necessary to understand these processes.

Assessment of Function within Closely Linked Opposite Pathways

of Elemental Cycling

1. Nitrogen Cycle

Microbes play an important role in the global cycling of nitrogen. Denitrifying bacteria reduce nitrate to NO_2^-, nitrous oxide, and eventually, to dinitrogen. Nitrogen lost by denitrification must be resupplied to the biosphere, either by atmospheric electrical discharge, or nitrogen fixation by microbes. Nitrogen fixation is extremely important in certain terrestrial (e.g., nitrogen-fixing nodules of legumes) and aquatic (e.g., heterocyst-forming cyanobacteria) habitats.

Although N seems to limit production in the sea, only a few organisms have been identified as nitrogen fixers. Several organisms (both free-living and symbiotic) potentially can fix N_2, but such activity may not have been detected because of methodological difficulties. Furthermore, nitrogen fixation in several marine environments has been debated, due to possible artifacts generated by the methods of detection (e.g., in organic-rich sediments). The potential for N-fixation in these organisms and environments can be determined directly by detecting the genes responsible for N-fixation (nif genes). Oligonucleotide primers have been successfully used to amplify (using PCR), clone, and sequence nif genes from DNA from a variety of microorganisms and natural environments, yielding information on the taxonomic identity of the nitrogen-fixing microorganism.

The use of an immunological probe in addition to a DNA probe in samples from a natural community to detect the presence of nitrogenase provides information on the expression of these genes. Such an approach could yield answers to the following questions: What is the distribution and diversity of nitrogen-fixing microorganisms? What environmental factors regulate the expression of nitrogenase? Are the numbers of N-fixing microorganisms consistent with our estimates of nitrogen fixation in the sea? More specifically, probes are available to show whether there is a potential for nitrogen-fixation in either the picoplankton, in the bacteria, or in the cyanobacteria component of the community.

Because nitrifiers and denitrifiers utilize the products of one reaction series as substrates for the opposite series, they

conspire to constrain the total amount of nitrogen available in the environment and its chemical composition. Thus, the degree of coupling between nitrification and denitrification may control the distribution of trace gases such as NO and N_2O, and, in balance with nitrogen fixation, may limit total global productivity by limiting the availability of fixed nitrogen. Autotrophic, rather than heterotrophic, nitrifiers make the major impact on nitrogen cycling in the environment, and by virtue of their highly specialized metabolism (obligately chemolithotrophic), they are a small, but phylogenetically diverse, group. Even though they comprise a very minor percentage of the total community, species-specific immunological probes and nucleic acid probes for individual strains are likely to be useful tools in quantifying and identifying nitrifying bacteria in complex assemblages. In contrast, denitrifiers are a much more diverse group, largely because the ability to denitrify is widespread and is insufficient to identify a metabolically coherent group. In this case, probes for functional entities, such as particular denitrifying enzymes and cofactors, will be more useful. Since denitrification, unlike nitrification, is an inducible metabolism, probes for gene expression and enzyme activity will be important in distinguishing the presence of the organism from the actual activity of the induced metabolism. Nitrifiers and denitrifiers occur as membranes of complex communities, on both large (oceanic oxygen-minimum zones) and small (sediment surface communities in shallow coastal environments) scales. Probes for specific individual strains or gene expression on the single cell basis will be necessary to dissect the interactions of these bacteria (see microbial consortia below). In terms of limiting factors, the nitrification/denitrification couple is important because we need to know not only what environmental factors control these two processes themselves, but what controls the effect of their interaction on the larger ecosystem: What determines the degree of linkage between nitrification and denitrification, and therefore, the net flux and loss of fixed nitrogen from the system?

Organism Function in the Methane Cycle

Methane is a radiatively active atmospheric trace gas that is currently increasing at a rate of approximately 1% per year (Blake and Rowland, 1988). If this increase continues unabated, predictions have been made that the effect of methane on global

warming will be 25% that of CO_2 within the next 50 years (Dickinson and Cicerone, 1986; Mitchell, 1989). Recent evidence has suggested that much of the methane increase is due to biogenic methane released to the atmosphere. The release is a result of imbalance between production and consumption, and therefore, it is important to understand the global biological sources and sinks of methane. Both the producers and consumers of methane are strictly prokaryotic; methane is produced by the archaebacterial group, the methanogens, and consumed by the eubacterial group, the methanotrophs (Rudd and Taylor, 1980). Standard techniques for studying the rates of methane production and consumption have always been hampered by the inability to directly assess the populations and their response to environmental changes. This problem is particularly amenable to a molecular approach. Probes are now available or are being developed for both groups, and therefore, it should be possible to combine rate measurements with direct assessment of populations. A series of such studies, carried out in a cross-section of the types of habitats known to be important in methane emission to the atmosphere, could yield a broad understanding of this process. This system has the potential for an early warning indicator, because the response of these two populations to environmental change could be predicted and monitored.

Assessment of Function in Specific Ecosystems: Coastal/Shelf Ecosystem

High primary production is common with coastal/shelf ecosystems. In many regions, significant increases in primary production and biomass have occurred in parallel with long-term modification and natural perturbation of the environment. Large-scale changes in grazer communities, notably in dominant fish stocks, have also occurred. In addition, this environment is the site of significant long-term carbon burial into sediments, and strong coupling between both land/sea and sea/atmosphere. These processes within the coastal and shelf ecosystem are of major consequence to global biogeochemical processes.

A fundamental question is the extent to which environmental perturbations, including long-term increases in nutrients, are progressively altering primary production, causing community reorganization and altered dynamics and energy flow at the primary

and upper trophic levels. In about 12 of the 20 major coastal systems globally, the dominant fish species has been replaced by another during the past decade. These major changes cannot presently be attributed to long-term changes in the environment or to fishing pressure. Coastal waters globally are also showing an increased frequency of blooms of novel species of noxious and toxic algae, which result in anoxia and large-scale deaths at all trophic levels -- events which increase carbon burial rates. Major questions which have emerged are: To what extent is the altered productivity and restructuring of the phytoplankton community a consequence of long-term chemical perturbation, of altered biotic interactions associated with increased toxic phytoplankton bloom, and of changing fish stocks and associated grazing pressure? The increased occurrence of noxious blooms suggests the following hypotheses: coastal environments are undergoing a functional group shift from diatom to flagellates; the selection vector is predominantly a response to an unknown stress, triggering genetic selection; the group selected is better adapted to the environmental perturbation, and is competitively favored in exploiting resources, although its suitability as prey and allelochemical potential may contribute to functional instability, community disequilibrium, and in the extreme case, to community dysfunction.

There are considerable quantitative data on the taxonomic structure, rates, and routes of carbon fixation, flow, and trophodynamics for a variety of coastal and shelf ecosystems. We must now establish the selective mechanisms by which species and functional groups within each trophic level are selected from the range of potentially available genetic stocks, i.e., genetic diversity; how this selection influences, and varies with, population growth, development, and the processes linking the various trophic levels; and how these linkages influence whole community properties such as succession, diversity, resilience, and equilibrium. We must seek to identify molecular and cellular indices of stress which may serve as an early warning (signal) and predictor of subsequent changes in community structure, function, and dynamics; and to assess predator-prey interaction at the molecular level, and allelochemical determinants of community organization and processes.

Gene Transfer in the Environment

Mobile genetic elements represent potential agents of change and adaptation in ecological communities. Little is known about the probability of interspecific and intraspecific gene transfer *in situ*, and the resultant effects on communities. The tools of molecular biology -- such as PCR, cloning and sequencing, hybridization technology, denaturing gradient gel electrophoresis -- offer the ability to identify and quantify gene transfer *in situ*. Combined with ecological investigations, these approaches may yield an understanding of the contribution of gene transfer to fitness (and niche), community stability, and adaptation to stress or change.

In situ conjugal transfer of bacterial genes has been well documented, for both aquatic and terrestrial ecosystems. This mechanism of transfer is thought to be facilitated at interfaces, e.g., in mat communities and in particulate consortia, where conditions for cell to cell contact are optimal. The existence of *in situ* transformation has long been suspected, but unequivocal demonstration is much more problematic. Free DNA exists in freshwater, marine, and terrestrial habitats, but its ultimate fate (i.e., impact on the gene pool) is unknown.

Recent observations revealed a high abundance of bacteriophages in the marine pelagic environment. Potentially, phages can mobilize bacterial genes by transduction, and so affect bacterial activities. The use of molecular techniques makes the estimation of the frequency of special types of phages and the occurrence of transducing particles an approachable problem. Moreover, specific probes can be developed to detect and enumerate phage particles, even if a host cell is unidentified and there is no way of propagating progeny phages. With such probes, the dynamics of viruses *in situ*, and their temporal and seasonal fluctuations, may be correlated with ecosystem biogeochemical cycling and activities.

Aquatic free phages are poorly defined, and no representative has yet been cloned, purified, or characterized. Phages of freshwater cyanobacteria and green algae are being intensively investigated, and these studies can guide research on the marine phages. Cloned and purified marine cyanobacterial phages can be characterized by determining their morphology, DNA mass, and host range. The replication of cyanobacterial phages closely resembles that of *E. coli* phages except that the times of each developmental

stage are markedly prolonged, which is probably associated with the intrinsically slower division rate of natural microbial hosts.

In principle, the development of rapid DNA sequencing techniques permits the determination of the entire genome of the free-living phages. A complete sequence of a phage will provide information on promoter sequences, termination signals, and ribosome binding sites of the phage, and, by extension, the host DNA.

Appendix IV
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